

# Hypertriglyceridaemia and low plasma HDL in a patient with apolipoprotein A-V deficiency due to a novel mutation in the *APOA5* gene

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**Abstract.** Priore Oliva C, Carubbi F, Schaap FG, Bertolini S, Calandra S (University of Modena & Reggio Emilia, Modena, Italy; AMC Liver Center, Amsterdam, The Netherlands; University of Genova, Genova, Italy). Hypertriglyceridaemia and low plasma HDL in a patient with apolipoprotein A-V deficiency due to a novel mutation in the *APOA5* gene (Case Report). *J Intern Med* 2008; **263**: 450–458.

*APOA5* encodes a novel apolipoprotein (apo A-V) which appears to be a modulator of plasma triglyceride (TG). In *apoA5* knock out mice plasma TG level increases almost fourfold, whereas in human *APOA5* transgenic mice it decreases by 70%. Some SNPs in the *APOA5* gene have been associated with variations in plasma TG in humans. In addition, hypertriglyceridaemic (HTG) patients have been identified who carried rare nonsense mutations in the *APOA5* gene (Q139X and Q148X), predicted to result in apo A-V deficiency. In this study we report a 17-year-old male with high TG and low high density lipoprotein chole-

sterol (HDL-C), who at the age of two had been found to have severe HTG and eruptive xanthomas suggesting a chylomicronaemia syndrome. Plasma postheparin LPL activity, however, was normal and no mutations were found in *LPL* and *APOC2* genes. The sequence of *APOA5* gene revealed that the patient was homozygous for a point mutation (c.289 C>T) in exon 4, converting glutamine codon at position 97 into a termination codon (Q97X). Apo A-V was not detected in patient's plasma, indicating that he had complete apo A-V deficiency. The administration of a low-fat and low-oligosaccharide diet, either alone or supplemented with  $\omega$ -3 fatty acids, started early in life, reduced plasma TG to a great extent but had a negligible effect on plasma HDL-C. Loss of function mutations of *APOA5* gene may be the cause of severe HTG in patients without mutations in *LPL* and *APOC2* genes.

**Keywords:** apolipoprotein A-V deficiency, high density lipoproteins, hypertriglyceridaemia, lipoprotein lipase.

## Introduction

Hypertriglyceridaemia (HTG) usually refers to fasting plasma triglyceride (TG) level above the 95th percentile for age and sex; it can be divided into primary and secondary type. Primary HTG is assumed to have a strong genetic basis; however, the molecular defect underlying primary HTG is known in <10% of cases [1].

Hyperlipidaemia type I and hyperlipidaemia type V are lipoprotein disorders characterized by the pathological presence of chylomicrons in the fasting state. Their clinical features include eruptive xanthomas, lipaemia retinalis, hepato-splenomegaly, recurrent abdominal pain and increased risk of pancreatitis [2]. The accumulation of chylomicrons (type I) or chylomicrons and VLDL (Type V) is caused by defects in the lipolytic cascade due to loss of function mutations

in the genes encoding lipoprotein lipase (*LPL*) or its activator apo C-II (*APOC2*) [2].

Familial HTG (hyperlipoproteinaemia type IV) is characterized by an isolated elevation of VLDL. This disorder has a population prevalence of 5–10% but its molecular basis is still unknown; it is likely to be polygenic and strongly affected by environmental factors and metabolic stress [1].

In familial combined hyperlipidaemia (FCHL) the elevation of plasma TG is due to increased level of VLDL, which is associated with increased level of LDL and hepatic overproduction of apolipoprotein B [3]. The molecular basis underlying FCHL is largely unknown; genetic variants in *APOA1/C3/A4/A5* cluster and in the recently identified *USF1* gene appear to contribute to the phenotype [4, 5].

The increased TG levels found in dysbetalipoproteinaemia (hyperlipoproteinaemia type III) is due to the accumulation of TG-rich lipoprotein remnants (e.g.  $\beta$ -VLDL). Patients with this disorder are typically homozygous for the rare apo E-2 isoform, which shows a defective binding to LDL-receptor when compared with the most common apo E-3 isoform. The phenotypic expression of dysbetalipoproteinaemia, however, requires some additional factors such as obesity, type 2 diabetes or hypothyroidism [3].

The recently identified *APOA5* gene, which encodes apolipoprotein A-V (apo A-V), appears to be a key modulator of plasma TG homeostasis in rodents as well as in humans. When the human *APOA5* gene was expressed in transgenic mice, the plasma TG level decreased by 70%, whereas when mouse *apoA5* gene was inactivated, the plasma TG increased four-fold, compared with control mice [6]. These results suggested an inverse correlation between apo A-V concentration and plasma TG level in mice. Single nucleotide polymorphisms in the *APOA5* gene (e.g. 1131T/C in the 5'-flanking region and c.56 C/G in the coding region) have been associated with differences in plasma TG levels in humans [6–10]. Carriers of the rare alleles –1131C or c.56 G have higher plasma TG than carriers of the corresponding common

alleles [7–10]. Interestingly, human studies do not support the inverse correlation between plasma apo A-V and TG concentration that has emerged from studies in mice. In fact, plasma apo A-V level was found to be positively correlated with TG level in subjects from the general population, as well as in patients with HTG and in patients with type 2 diabetes [11–16].

Rare loss of function mutations in *APOA5* gene have been reported in humans [17]. In 2005 we identified a 9-year-old boy with severe HTG, who was homozygous for a nonsense mutation predicted to result in truncated apo A-V (Q148X) [18]. He was regarded to have complete apo A-V deficiency as no apo A-V was detected in his plasma [17]. Another patient with type V hyperlipidaemia and recurrent episodes of pancreatitis was found to be homozygous for a different apo A-V mutation (Q139X) predicted to cause complete apo A-V deficiency [19]. So it looks as though apo A-V deficiency in humans recapitulates, to some extent, the plasma lipid abnormalities observed in *apoA5* knock-out mice, suggesting that the absence of apo A-V causes a substantial disruption of the metabolism of TG-rich lipoproteins.

In the present study we report a 17-year-old patient with primary HTG detected early in life, who was found to be homozygous for a novel nonsense mutation of *APOA5* gene. No apo A-V was detectable in his plasma, thus suggesting that he has complete apo A-V deficiency.

## Methods

### Subject

Proband T.L. is a 17-year-old Caucasian male, who at the age of two was found to have eruptive cutaneous xanthomas and severe HTG in the fasting state, suggesting hyperchylomicronaemia syndrome. He never had abdominal pain, hepatosplenomegaly nor pancreatitis and developed normally. Routine laboratory tests, with the exception of plasma lipids, were within the reference range. The proband has been treated with low-fat and low-oligosaccharide diet since infancy;

the good compliance to this diet was associated with a substantial reduction of plasma TG level (Table 1) and a progressive disappearance of eruptive xanthomas. At the age of 12 he started treatment with  $\omega$ -3 fatty acids (3 g day<sup>-1</sup>) which, however, was discontinued after 2 years because of poor compliance. Medium Chain Triglycerides (MCT) supplementation or fibrate therapy were never prescribed.

At age 16 the proband was referred to the Lipid Clinic of the University Hospital with the clinical diagnosis of primary severe HTG (type V). Alcohol assumption was denied. He was a nonsmoker and practised regular physical activity (basketball player). On physical examination he was found healthy and lean (BMI 19.5 kg m<sup>-2</sup>, waist circumference 71 cm). He had normal blood pressure and heart rate; no skin, tendons, thoracic, abdominal or vascular abnormalities were observed. Tonsils, eyes and fundus oculi examinations were normal. Abdominal ultrasound scan confirmed the absence of visceral abnormalities. Routine laboratory tests for hepato-biliary, renal and

thyroid function were normal, as well as blood glucose and blood cell count. ECG was normal.

On a low-fat diet, the level of total cholesterol (T-C), TG and HDL cholesterol (HDL-C) were 4.08, 5.90 and 0.75 mmol L<sup>-1</sup>, respectively (Table 1). The addition of  $\omega$ -3 fatty acids (3 g day<sup>-1</sup>) lowered triglycerides to 2.98 mmol L<sup>-1</sup> but had little effect on plasma HDL-C (Table 1).

Proband's parents were apparently unrelated; they were in good health and had no family history of dyslipidaemia or premature coronary heart disease. The proband's 7-year-old brother was also in good health. Routine laboratory tests of these family members were within the normal values (Table 2).

#### Plasma lipid analysis and LPL assay

T-C, HDL-C, TG, apo A-I and apo B levels were determined by standard methods and plasma lipoprotein were separated by density gradient ultracentrifugation in a SW41 Beckman rotor at 170 000  $\times g$  at 15 °C [20]. The measurement of plasma apo A-V and apo C-III was performed by ELISA assay; immunoblot analysis of plasma apo A-V was conducted as described [11]. Lipoprotein and hepatic lipase activities in postheparin plasma were measured as previously reported [18].

#### Analysis of candidate genes

Genomic DNA was extracted from peripheral blood leucocytes by a standard procedure. All exons and the promoter region of *LPL* and *APOC2* genes were amplified by polymerase chain reaction from genomic

**Table 1** Plasma lipids and lipoproteins in proband T.L.

Years of age	Treatment	TG	T-C	HDL-C	LDL-C	Apo B	Apo A-I
2	None	9.65	5.58	0.59	n.d.	n.d.	n.d.
3	Diet	2.82	4.73	0.58	n.d.	n.d.	n.d.
4	Diet	3.59	5.68	0.80	1.47	175	118
5	Diet	5.13	4.60	0.59	n.d.	n.d.	n.d.
10	Diet	3.61	3.18	0.67	1.49	123	173
11	Diet	5.08	4.34	0.88	n.d.	n.d.	n.d.
12	Diet + $\omega$ -3	3.73	4.48	0.90	n.d.	n.d.	n.d.
13	Diet + $\omega$ -3	2.37	3.18	0.64	n.d.	n.d.	n.d.
13	Diet	4.02	4.37	0.82	1.57	102	126
14	Diet	5.28	4.19	0.69	n.d.	n.d.	n.d.
15	Diet	12.03	4.96	0.59	n.d.	n.d.	n.d.
16	Diet	5.90	4.08	0.75	1.73	n.d.	n.d.
17	Diet + $\omega$ -3	2.98	3.93	0.75	1.68	80	112

Plasma level of TG, T-C, HDL-C and LDL-C are given in mmol L<sup>-1</sup>; plasma level of apo B and apo A-I are given in mg dL<sup>-1</sup>.

Diet +  $\omega$ -3 fatty acids 3 g day<sup>-1</sup> (oral ethyl-ester of polyunsaturated fatty acids EPA and DHA 85%, EPA/DHA = 0.9–1.5).

n.d., not determined.

**Table 2** Plasma lipids and lipoproteins in proband's family members

Subject	TG	T-C	HDL-C	LDL-C	Apo B	Apo A-I
Father	0.79	5.85	1.26	4.54	128	154
Mother	1.27	4.25	1.21	2.86	78	157
Brother	0.56	3.69	1.03	2.74	68	127

Plasma level of TG, T-C, HDL-C and LDL-C are given in mmol L<sup>-1</sup>; plasma level of apo B and apo A-I are given in mg dL<sup>-1</sup>.

DNA and sequenced as described [18]. The *APOA5* gene was amplified and sequenced as reported [18]. The mutation of *APOA5* gene found in the proband, which eliminates a *PstI* restriction site in exon 4, was confirmed by restriction fragment analysis of PCR-amplified 5' region of exon 4, using the following primers: 5'-CGG CCT GGA TAT CTG TCC CC-3' (forward primer) and 5'-CCC AGT GCC TGC AAA GGC TC-3' (reverse primer). The amplification conditions were 96 °C for 5 min, 96 °C for 1 min per 61.1 °C for 1 min per 72 °C for 1 min for 35 cycles, followed by 72 °C for 5 min. The amplification product (662 bp) was digested with *PstI* and the fragments were separated by 2% agarose gel electrophoresis. This procedure was used to screen the mutation in 100 randomly selected normolipidaemic subjects.

Nomenclature of the mutation was according to the recommendations of the Human Genome Variation Society (the numerical series of codons includes the sequence of the signal peptide, <http://www.hgvs.org/mutnomen>).

The proband and family members were also genotyped for the following polymorphisms: -482 C/T in *APOC3* gene;  $\epsilon 2$ ,  $\epsilon 3$  and  $\epsilon 4$  in *APOE* gene [19]. The polymorphisms of *APOA5* gene were assessed by direct sequencing.

### Ethical aspects

Informed consent was obtained from the patient and his family and the study protocol was approved by the local ethical committee of the University Hospital of Modena.

## Results

### Plasma lipid and lipoproteins

The plasma lipid levels of the proband, recorded since 2 years of age, are shown in Table 1. Plasma TG level shows large fluctuations over time (especially in early teens), mostly in relation to the compliance of the patient to the low-fat and low-oligosaccharide diet and treatment with  $\omega$ -3 fatty acids. Plasma HDL-C

levels showed minor changes and were less affected by dietary treatment. Plasma lipoprotein profile performed when the patient's TG level was 5.90 mmol L<sup>-1</sup> showed a marked increase of the peak corresponding to lipoproteins of density <1.030 g mL<sup>-1</sup> (Chylomicrons, VLDL + IDL) and a reduction of LDL and HDL peaks (data not shown). Plasma postheparin LPL activity (9.5  $\mu$ mol FFA mL<sup>-1</sup>) was within the control range (8–15  $\mu$ mol FFA mL<sup>-1</sup>). The *APOE* genotype of the proband was  $\epsilon 3\epsilon 4$ . The parents and the brother of the proband were found to have normal lipid levels (Table 2).

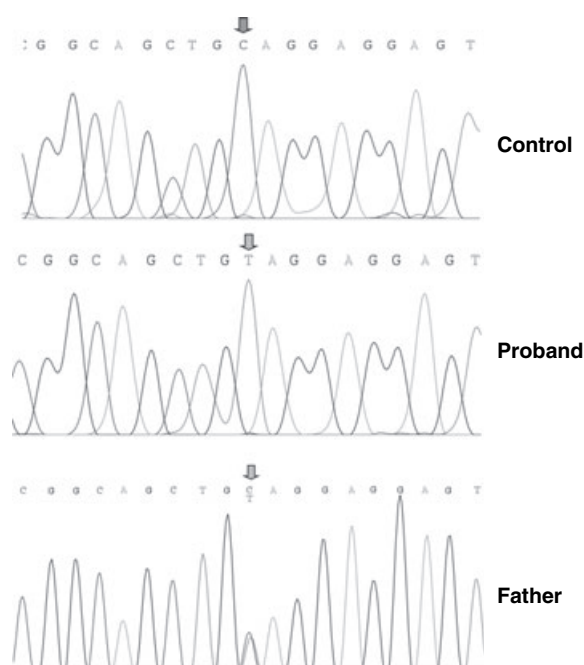
### Sequence of *APOA5* gene

Since the sequence analysis of *LPL* and *APOC2* genes in the proband revealed no abnormalities, we sequenced his *APOA5* gene. This analysis revealed that the proband was homozygous for a single nucleotide substitution in exon 4 (c.289 C>T), which converts the glutamine codon at position 97 into a termination codon (Q97X) (Fig. 1). The presence of this mutation was confirmed by restriction fragment analysis after digestion of exon 4 with the restriction enzyme *PstI*, one site of which is abolished by the mutation (Fig. 2).

The parents and the brother of the proband were found to be heterozygous for the mutation (Figs 1 and 2). *APOA5* gene polymorphisms in the proband's family are shown in Table 3. No carrier of the mutation was found amongst 100 randomly selected normolipidaemic individuals, nor in 30 patients with primary severe HTG negative for mutations in *LPL* or *APOC2* genes. The proband was homozygous for the common allele in the promoter of *APOC3* gene (-482 C/C), whilst his parents were heterozygous (-482 C/T).

### Plasma apo A-V and apo C-III levels

Apo A-V was undetectable in proband's plasma; the level of apo A-V in proband's father (26.9  $\mu$ g dL<sup>-1</sup>) was within the control range (25.8  $\pm$  14.6  $\mu$ g dL<sup>-1</sup>). Immunoblot analysis of proband's plasma failed to



**Fig. 1** Partial nucleotide sequence of exon 4 of *APOA5* gene in a control subject, in proband T.L. and in his father. The proband is homozygous for a C>T transition (c.289 C>T) converting the glutamine codon at position 97 into a termination codon (Q97X). Proband's father was heterozygous for the same mutation.

reveal the presence of either wild type or truncated apo A-V (Fig. 3). Plasma apo C-III level in the proband was  $18.4 \text{ mg dL}^{-1}$  and  $10.8 \text{ mg dL}^{-1}$  in his father (in fasting normolipidaemic subjects  $10.6 \pm 3.6 \text{ mg dL}^{-1}$ ).

## Discussion

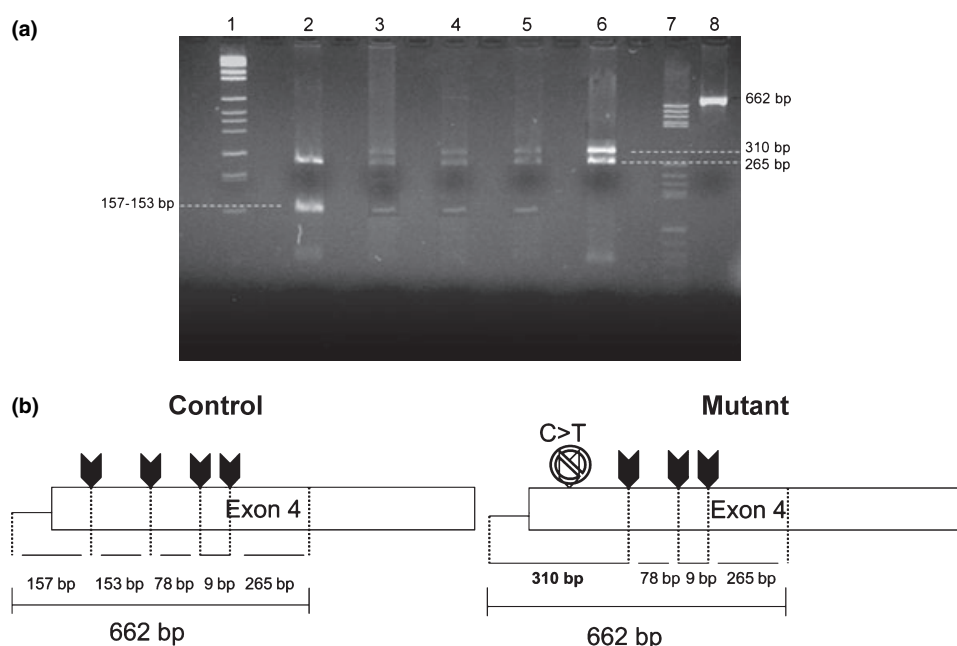
In this study we report a novel mutation in *APOA5* gene which causes a complete apo A-V deficiency. This mutation was discovered during a survey of 48 patients with severe primary HTG, suspected to have genetic defects in the lipolytic cascade. Amongst the HTG patients found to be negative for mutations in the *LPL* and *APOC2* genes (30 subjects) we identified three patients who carried pathogenic mutations of *APOA5* gene. The clinical and biochemical features of the first two patients were described previously [18, 21]. The patient investigated in the present study was found to have eruptive xanthomas and milky

plasma in the fasting state at the age of 2 years, suggestive of primary hyperchylomicronaemia syndrome. He was put on a restricted dietary regimen (low-fat and low-oligosaccharide diet) which was effective in reducing plasma TG and eliminating eruptive xanthomas. Additional benefits were obtained with supplementation of  $\omega$ -3 fatty acids. During the 15 years of follow-up plasma TG exceeded the critical level of  $10 \text{ mmol L}^{-1}$  (regarded to be the threshold for the risk of pancreatitis) only on one occasion. It is most likely that the strict dietary regimen, the regular physical activity during adolescence and the maintenance of an ideal BMI have contributed to prevent excessive elevations of plasma TG over the years. The normal postheparin LPL activity (performed at the age of 12) cast doubt on the presence of defects in the lipolytic cascade and the patient was referred to the laboratory for molecular diagnosis at the age of 17. After the exclusion of *LPL* and *APOC2* genes, the analysis of *APOA5* gene showed that proband was homozygous for a point mutation in *APOA5* gene, predicted to result in a short truncated protein (Q97X). Neither wild type nor truncated apo A-V was detected in proband's plasma, thus confirming that the patient had a complete apo A-V deficiency. The truncated protein Q97X, if synthesized at all, is expected to be completely devoid of function, as it lacks some domains involved in lipid binding (residues 192–238 and 301–343), LPL activation (residues 192–238) and heparin binding (residues 186–227) [22–24].

Phenotypically proband T.L. resembles the patient with complete apo A-V deficiency who was homozygous for the truncated apo A-V Q148X we reported previously [18]. Both these young patients had severe HTG since infancy, which was transmitted as a recessive trait in the family. In both patients the administration of a low-fat diet, especially if supplemented with  $\omega$ -3 fatty acids, induced a substantial reduction of plasma TG level (Table 4).

Although the mechanism by which apo A-V affects plasma TG level is not fully understood, several hypotheses have been proposed including: (i) an intracellular effect on hepatic VLDL assembly and secretion (apo A-V would down-regulate the assembly and





**Fig. 2** Restriction fragment analysis of exon 4 of *APOA5* gene. Exon 4 was amplified by PCR and digested with the restriction enzyme *PstI*. The lower panel shows the restriction fragments obtained in a control subject and in proband T.L., in whom the c.289 C>T substitution abolishes a *PstI* restriction site at the 5' end of exon 4. The sizes of the restriction fragments are indicated below the diagram. The upper panel shows the separation of the restriction fragments by 2% agarose gel electrophoresis. Lanes 1 and 7: molecular weight markers; lane 2: control subject; lanes 3–5: mother, father and brother of the proband; lane 6: proband T.L.; lane 8: undigested PCR fragment of exon 4.

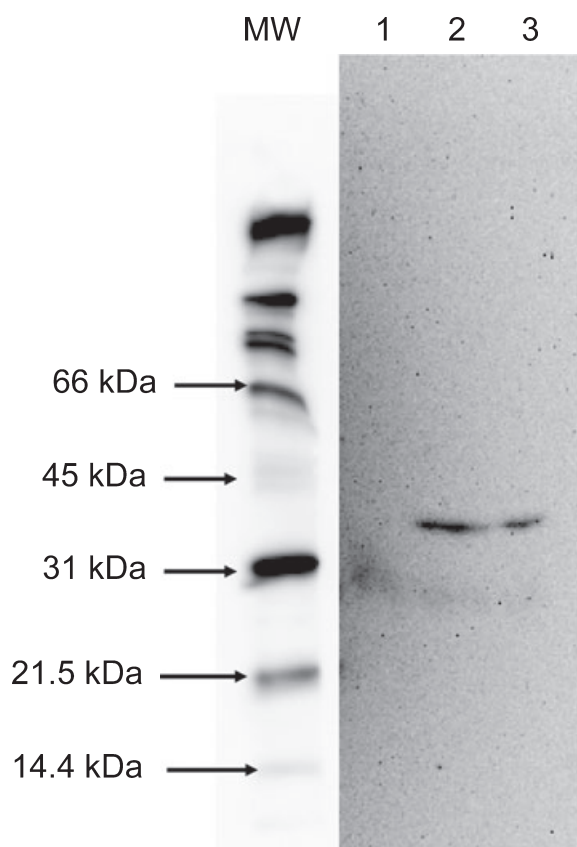
**Table 3** *APOA5* gene polymorphisms in proband T.L family

	–1131T>C (SNP3)	c.-3A>G	c.56C>G (S19W)	IVS3 + 476G>A (SNP2)	c.1259T>C (SNP1)
Subject					
Proband	T/T	A/A	C/C	G/G	T/T
Father	T/T	A/A	C/C	G/G	T/T
Mother	T/T	A/A	C/C	G/G	T/T
Brother	T/T	A/A	C/C	G/G	T/T

secretion of VLDL) and (ii) an effect on plasma removal of TG-rich lipoprotein, either by a direct modulation of the activity of lipoprotein lipase or by an effect on the uptake of these lipoproteins (or their remnants) by hepatocytes [24].

Some studies indicate that apo A-V facilitates the action of LPL. In view of its high affinity for heparin, apo A-V would increase the binding of TG-rich lipoproteins to glycosaminoglycans present on the

surface of endothelial cells, thus rendering these lipoproteins more accessible to LPL [25]. Recently, Nilsson *et al.* [26] provided evidence that apo A-V binds to two endocytotic receptors of the LDLR family, LRP and SorLA, and mediates the binding of chylomicrons to these receptors. They also put forward the hypothesis that the main action of apo A-V is exerted at the sinusoidal surface of the hepatocytes rather than in the plasma compartment. Apo A-V (which is synthesized exclusively in the liver)



**Fig. 3** Immunoblot analysis of plasma apo A-V. Plasma proteins were separated on 10% SDS-PAGE and transferred to PVDF membranes. Apo A-V was detected using rabbit polyclonal antibodies directed against apo A-V and appropriate secondary antibody. Signals were visualized by chemiluminescence. Five microlitre of plasma was applied to each lane. Lane 1: proaband T.L.; lane 2: proaband's father; lane 3: control subject. Molecular weight markers are shown on the left.

secreted by the hepatocytes would be entrapped by heparan-sulphate proteglycans and endocytotic receptors present at the cell surface [27]. In this way apo A-V would facilitate lipoprotein uptake (i.e. chylomicron remnants removal). These results are in line with the observation that the clearance of chylomicron remnants is reduced in *apoa5* knock-out mice [28]. In this context one might assume that in patients with apo A-V deficiency the LPL-mediated TG hydrolysis of chylomicrons and VLDL is less efficient and the clearance of lipoprotein remnants by the liver is delayed. The magnitude of the accumula-

tion in plasma of TG-rich lipoproteins in these patients is probably related to the amount of these lipoproteins entering the plasma compartment. Excessive secretion of chylomicrons due to a high fat intake, or excessive secretion of VLDL by the liver induced by various metabolic stimuli, may overload the less efficient LPL-mediated hydrolysis of TG-rich lipoproteins and impair the capacity of the liver to capture lipoprotein remnants, in case of apo A-V deficiency. This may explain why in our two patients with apo A-V deficiency (Q97X and Q148X) dietary interventions, aimed at reducing chylomicron formation (reduced oral fat load) and/or VLDL synthesis and secretion (administration of  $\omega$ -3 fatty acids) [29], have such a profound TG-lowering effect.

The other remarkable finding in proaband T.L. is the persistently low level of plasma HDL-C, which was only marginally affected by dietary treatment. The hypothesis that the absence of apo A-V, which is a minor protein component of HDL, renders HDL more unstable and more easily removed from the circulation, is unlikely in view of the minute amounts of apo A-V present in human plasma. It is more likely that the delayed TG hydrolysis, due apo A-V deficiency *per se* or due to the increased level of apo C-III (an inhibitor of LPL) [30], reduces the availability of surface components of TG-rich lipoproteins, which contribute to HDL formation. It was recently shown that adenovirus mediated delivery of apoA-V into HTG APOC3 transgenic mice not only reduced triglyceride and VLDL-C level but also increased HDL-C level with an increase of both the number and size of HDL particles [31]. These data suggest that in addition to its TG-lowering effects, apoA-V plays a significant role in modulating HDL maturation and cholesterol metabolism.

The three heterozygotes for Q97X mutation investigated in this study (Table 2) have normal plasma TG, suggesting that partial apo A-V deficiency is not sufficient *per se* to induce HTG and other predisposing factors are required. We previously reported that only five out of ten carriers of Q148X mutation had moderate elevation of plasma TG ( $>2$  mmol L<sup>-1</sup>; the

**Table 4** Main clinical features of the patients homozygous for nonsense mutations of *APOA5* gene

ApoA-V mutation	Plasma TG mmol L <sup>-1</sup>	Plasma apoA-V	Pancreatitis	<i>APOE</i> genotype	Reference
Q97X	9.65	undetectable	No	ε3/ε4	present study
Q139X <sup>a</sup>	15–30	n.d.	Yes	ε3/ε3	[19]
Q148X	50.16	undetectable	No	ε3/ε3	[17, 18]

nd, not determined.

<sup>a</sup>Deceased at the age of 60 from a cause unrelated to dyslipidaemia [19]

highest level observed was 4.65 mmol L<sup>-1</sup>). Carriers with elevated plasma TG tended to be older, to have higher body mass index and higher fasting plasma glucose level than normotriglyceridaemic carriers [18]. Amongst the carriers of Q139X belonging to one of the families reported by Marcais *et al.* [19], two had severe HTG and four had plasma TG levels of 2 mmol L<sup>-1</sup> or less. The two individuals with severe HTG had diabetes and low LPL activity.

In conclusion, we report the third mutation in *APOA5* gene that results in apo A-V deficiency in a patient with primary HTG. We also confirmed that strict dietary treatment and supplementation with ω-3 fatty acids induce a substantial reduction of plasma TG level.

We predict that the systematic analysis of the major candidate genes controlling the lipolytic cascade in patients with primary HTG will lead to the discovery of other loss of function mutations of *APOA5* gene.

### Conflict of interest

No conflict of interest was declared.

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